Retinoid-related orphan nuclear receptor RORβ is an early-acting factor in rod photoreceptor development

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Rods and cones are morphologically and developmentally distinct photoreceptor types with different functions in vision. Cones mediate daylight and color vision and in most mammals express M and S opsin photopigments for sensitivity to medium-long and short light wavelengths, respectively. Rods mediate dim light vision and express rhodopsin photopigment. The transcription factor networks that direct differentiation of each photoreceptor type are incompletely defined. Here, we report that *Rorb*$^{-/-}$ mice lacking retinoid-related orphan nuclear receptor β lose rods but overproduce primitive S cones that lack outer segments. The phenotype reflects pronounced plasticity between rod and cone lineages and resembles that described for *Nrl*$^{-/-}$ mice lacking neural retina leucine zipper factor. *Rorb*$^{-/-}$ mice lack *Nrl* expression and reexpression of *Nrl* in *Rorb*$^{-/-}$ mice converts cones to rod-like cells. Thus, *Rorb* directs rod development and does so at least in part by inducing the *Nrl*-mediated pathway of rod differentiation.

cone | differentiation

Rods and cones are distinct receptor cell types that mediate dim and bright light vision, respectively. In the mouse, cones are generated between midgestation and birth (1) and subpopulations differentially express M and S opsin photopigments for sensitivity to medium-long and short light wavelengths, respectively (2). Rods express rhodopsin and greatly outnumber cones in mice. Rod generation lags behind that of cones and is more protracted, lasting until about a week after birth. Rods, cones, and other retinal cell types are generated in a stereotypical order from multipotent progenitors, and it has been proposed that a combination of transcription factor activities and external signals at a given developmental stage prompts progenitors to enter specific differentiation pathways (3, 4).

The transcription factors that direct the generation of rod and cone precursors and the terminal differentiation of these cell types are incompletely defined. During terminal differentiation of cones, thyroid hormone receptor TRβ2 is required for M opsin induction such that without TRβ2, cones express only S opsin (5). Factors that promote rod differentiation and survival include leucine zipper protein Nrl (6), orphan nuclear receptor Nr2e3 (7, 8), homeodomain proteins Crx and Otx2 (9–11), and retinoblastoma protein Rb (12, 13). *Nrl* induces *Nr2e3* expression and these two genes define a transcriptional hierarchy for rod differentiation. *Nrl*$^{-/-}$ mice overproduce S cones at the expense of rods (6) whereas ectopic *Nrl* expression converts cones to rods (14). *Nr2e3* deficiency causes an enhanced cone phenotype and misexpression of cone genes in rods, suggesting that *Nr2e3* represses cone genes to maintain the rod phenotype (15–19). Human *NRL* and *NR2E3* mutations result in retinopathy phenotypes (8, 20). The above findings suggest that rod and cone precursors share a default differentiation program as cones and that rod differentiation requires the action of additional transcription factors.

The *Rorb* gene encoding retinoid-related orphan nuclear receptor RORβ is expressed in the brain, pineal gland, and retina. *Rorb* is expressed in all neural retina layers from early stages with a peak at neonatal stages, suggestive of a role in many differentiating retinal cell types including both cones and rods (21–23). We previously reported that *Rorb* and *Crx* synergistically induce the S opsin promoter (24), indicating a role for *Rorb* in cone differentiation. Here, we report a role for *Rorb* in rods. We found that *Rorb*$^{-/-}$ mice overproduce cones at the expense of rods and lack *Nrl* and *Nr2e3* expression. Reexpression of *Nrl* in these mice converted the excess cones to rod-like cells. Thus, *Rorb* is critical for rod differentiation and lies upstream of *Nrl* in the rod transcriptional pathway.

Results

Loss of *Nrl*-Mediated Rod Differentiation Pathway in *Rorb*$^{-/-}$ Mice. *Rorb*$^{-/-}$ mice displayed gross overexpression of S opsin and severe loss of rhodopsin mRNA (Fig. 1*A*). A small number of rhodopsin-positive (rhodopsin^{+}) cells remained around the outer nuclear layer (ONL). Although overexpressed in *Rorb*$^{-/-}$ mice, S opsin followed a normal distribution gradient with stronger signals in the inferior (ventral) than superior (dorsal) retina (25). In wild type (+/+) mice, cones represent only 3% of all photoreceptors and reside near the outer edge of the ONL whereas rods represent 97% of photoreceptors and populate the entire depth of the ONL (26). In *Rorb*$^{-/-}$ mice at P23, S opsin^{+} cells populated the entire ONL. The extent of S opsin overexpression was emphasized by the fact that *Rorb*$^{-/-}$ mice possessed only approximately 40% of the number of photoreceptors found in +/+ mice (see Fig. 3*E*). A few displaced S opsin^{+} cells were detected in the inner nuclear layer (INL) in *Rorb*$^{-/-}$ mice. M opsin expression in *Rorb*$^{-/-}$ mice at P23 was slightly elevated in the superior retina but was absent in the inferior reflecting an exaggeration of the normal bias in distribution found in +/+ mice.

The similarity of the above phenotype to that of *Nrl*$^{-/-}$ mice suggested that in *Rorb*$^{-/-}$ mice, photoreceptor precursors that would normally form rods differentiated instead as S cones. S opsin overexpression in *Rorb*$^{-/-}$ mice occurred between P9 and P14 in a large, late-maturing population of photoreceptors (Fig. 1*B*). *Rorb*$^{-/-}$ mice also produced a small, early-appearing cone population like that of +/+ mice that expressed the early cone marker TRβ2 at E18.5 (see Fig. 2*C*). However, unlike in +/+ mice, this cone population in *Rorb*$^{-/-}$ mice failed to express S opsin mRNA or protein at P6 (Fig. 1 *B* and *C*) in accord with the previous proposal that *Rorb* is required with *Crx* to induce S opsin in normal cone development (24). Somewhat paradoxically, S opsin overexpression at later stages in the excess cones in *Rorb*$^{-/-}$ mice was independent of *Rorb*, suggesting that this is

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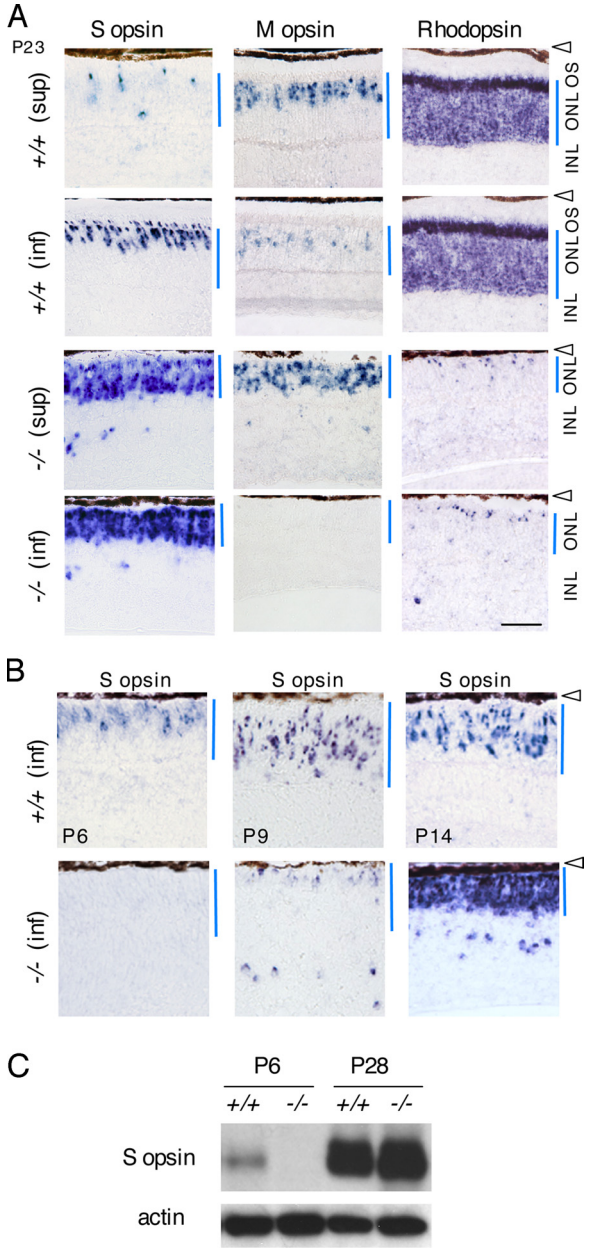

Fig. 1. Enhanced S opsin phenotype in *Rorb*$^{-/-}$ mice. (*A*) Overexpression of S opsin and loss of rhodopsin in *Rorb*$^{-/-}$ mice at P23 shown by in situ hybridization in superior (sup) and inferior (inf) retina. At P23, S opsin^{+} cones reside at the outer edge of the ONL (blue bar) in +/+ mice but populate the entire ONL in *Rorb*$^{-/-}$ mice. *Rorb*$^{-/-}$ mice lack outer segments (OS) and the ONL is adjacent to the retinal pigmented epithelium (RPE, arrowhead). In *Rorb*$^{-/-}$ mice, a few (approximately 200) residual rhodopsin^{+} cells were detectable per 10 μm thick cryosection. (*B*) Late onset of S opsin overexpression in *Rorb*$^{-/-}$ mice. Note that in +/+ mice, cones migrate across the nascent ONL toward the inner nuclear layer (INL) then return to the outer edge of the ONL to reach their mature location near weaning age. (*C*) Western blot showing lack of S opsin at P6 and overexpression at P28 in retina in *Rorb*$^{-/-}$ mice.

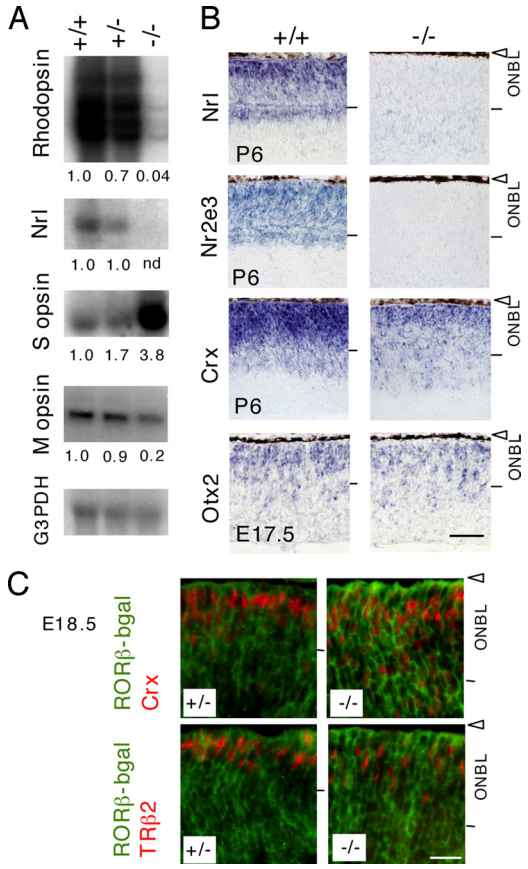

Fig. 2. Loss of *Nrl* and *Nr2e3* expression in *Rorb*$^{-/-}$ mice. (*A*) Northern blot showing loss of Nrl and rhodopsin and overexpression of S opsin mRNA in *Rorb*$^{-/-}$ mice at P14. Numbers below lanes, quantitation relative to +/+ samples, assigned a value 1.0 (normalized to G3PDH); n.d., not detectable. (*B*) In situ hybridization showing loss of *Nrl* and *Nr2e3* but retention of *Crx* and *Otx2* mRNA expression in *Rorb*$^{-/-}$ mice. Expression was analyzed in the first postnatal week (P6) when *Nrl*, *Nr2e3*, and *Crx* approach peak expression. *Otx2* was analyzed at E17.5 as peak *Otx2* expression in the ONBL occurs in the embryo. (*C*) Presence of Crx^{+} and TRβ2^{+} photoreceptor precursors in *Rorb*$^{-/-}$ embryos. Immunofluorescent detection of RORβ-β-galactosidase (nonnuclear, green cell bodies) expressed from *lacZ* knocked into *Rorb* (22) and Crx (red nuclei) a marker for rods and cones or TRβ2 (red nuclei) a marker for cones. (Scale bars, *B*, 50 μm, *C*, 20 μm.)

mediated by other means than in normal cone development. The findings indicate that *Rorb* mediates distinct functions in cone and rod precursors.

Northern blot, in situ hybridization, and Western blot analyses revealed loss of *Nrl* and *Nr2e3* expression in *Rorb*$^{-/-}$ mice (Figs. 2 *A* and *B* and 3*A*). However, *Rorb*$^{-/-}$ mice still expressed *Crx*, which is required for terminal differentiation of both rods and cones (9, 10), and *Otx2*, which induces *Crx* expression and participates in rod differentiation (11). The findings indicate that *Rorb* controls rod differentiation upstream of *Nrl* and *Nr2e3* but independently, or downstream, of *Otx2*. In situ hybridization and quantitative PCR did show reduced *Crx* expression in postnatal *Rorb*$^{-/-}$ pups (Fig. S1), a decrease that may be explained by the partial loss of photoreceptors, although it is not ruled out that *Rorb* is also necessary for full expression of *Crx*.

Immunostaining for Crx as a marker for all photoreceptors (rods and cones) or TRβ2 as a marker for cones indicated that *Rorb*$^{-/-}$ embryos produced photoreceptor precursors that would normally form both rods and cones (Fig. 2*C*). *Rorb* gene expression, revealed using an *Rorb-lacZ* knockin allele, was detected in almost all neural retinal cells, including both Crx^{+} and TRβ2^{+} cells. In *Rorb*$^{-/-}$ embryos, Crx^{+} cells and TRβ2^{+} cells were dispersed in both outer and inner neuroblastic layers, indicating early abnormalities in migration and retinal organization. Increased numbers of apoptotic cells were detected in the retina of *Rorb*$^{-/-}$ embryos and juvenile mice that could account for the ultimate reduction of photoreceptor numbers (Fig. S2).

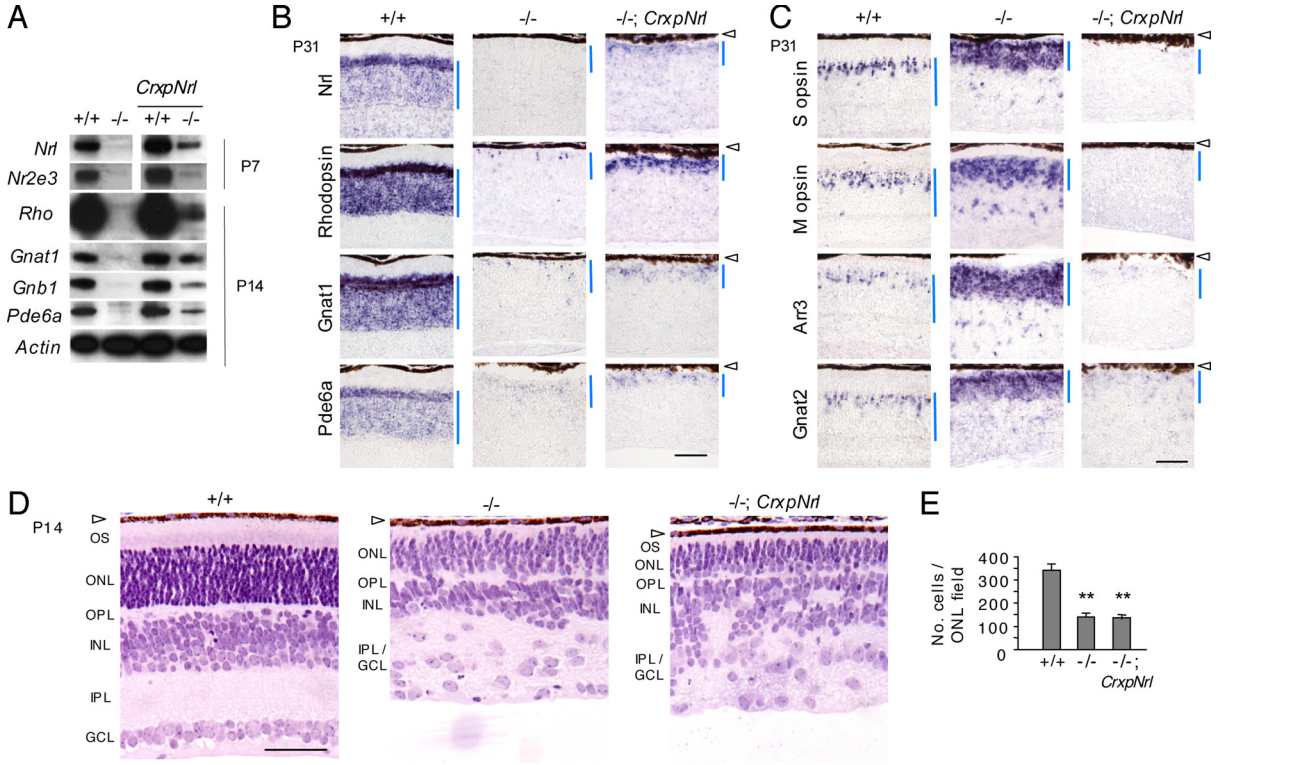

Fig. 3. Recovery of rod-like photoreceptors in *Rorb*$^{-/-}$;*CrxpNrl* mice. (*A*) Western blot analysis. The *CrxpNrl* transgene expresses Nrl and reinduces rod markers Nr2e3, rhodopsin, Gnat1, Gnb1,and Pde6a in *Rorb*$^{-/-}$ mice at ages indicated. (*B*) In situ hybridization showing partial rescue of rod gene expression in *Rorb*$^{-/-}$;*CrxpNrl* mice. In *Rorb*$^{-/-}$ mice, the ONL (blue bar) is adjacent to the RPE (arrowhead). (*C*) In situ hybridization showing suppressed cone gene expression in *Rorb*$^{-/-}$;*CrxpNrl* mice. (*D*) Methacrylate sections showing a thin ONL in *Rorb*$^{-/-}$ mice at P14. Compared to *Rorb*$^{-/-}$ mice, *Rorb*$^{-/-}$;*CrxpNrl* mice have a more organized ONL, rod-like photoreceptor nuclei and recovery of a small OS layer at P14. OPL, outer plexiform layer, IPL, inner plexiform layer, GCL, ganglion cell layer. (Scale bars, *C*, *D*, *E*, 50 μm.) (E) *Rorb*$^{-/-}$ and *Rorb*$^{-/-}$;*CrxpNrl* mice have approximately 40% of normal photoreceptor numbers. All photoreceptor (cone and rod) nuclei were counted in ONL fields on 3 μm sections at P14. Means $\pm$ SD; **, $P < 0.001$ versus +/+ mice.

***Nrl* Reexpression Partly Rescues Rod Development in *Rorb*$^{-/-}$ Mice.** To establish that *Rorb* acts upstream of *Nrl* in the same rod differentiation pathway, an *Nrl*-expressing transgene was introduced into *Rorb*$^{-/-}$ mice by crossing *Rorb*$^{-/-}$ mice with *CrxpNrl* transgenic mice. The *CrxpNrl* transgene carried a *Crx* promoter that is active in both cone and rod precursors from E12.5 onwards (27, 14). Moreover, expression of *Crx*, unlike many photoreceptor genes, was retained in *Rorb*$^{-/-}$ mice. *Rorb*$^{-/-}$ mice carrying the transgene (*Rorb*$^{-/-}$;*CrxpNrl*) were analyzed from early postnatal stages up to approximately 4 weeks of age, spanning the period of peak *Rorb* and *Nrl* expression in the first postnatal week (24, 28) and the subsequent terminal differentiation of rods in normal mice. Given the disorganization of the retina in adult *Rorb*$^{-/-}$ mice (22), older mice were not analyzed to avoid the possibility of degeneration obscuring the comparison of *Rorb*$^{-/-}$ and *Rorb*$^{-/-}$;*CrxpNrl* mice.

Rorb$^{-/-}$;*CrxpNrl* expressed Nrl protein at 51% of the level of +/+ mice at P7 as shown by Western blot analysis (Fig. 3*A*). The lower level may be explained by the lower photoreceptor number in *Rorb*$^{-/-}$ mice and by differences in the strength of the transgene promoter relative to the endogenous *Nrl* promoter. Most rod genes tested, including *Nr2e3*, *Rho*, *Gnat1*, *Gnb1* and *Pde6a* showed a degree of recovered expression in *Rorb*$^{-/-}$;*CrxpNrl* mice as detected by Western blot, in situ hybridization (Fig. 3*B*) and microarray (Fig. 5*A*) analyses. In +/+ mice, the *CrxpNrl* transgene suppresses cone genes suggesting that *Nrl* can inhibit all photoreceptor precursors, including those that would normally form cones, from acquiring cone properties (14). In *Rorb*$^{-/-}$;*CrxpNrl* mice, cone gene expression was strongly suppressed indicating that all cones in *Rorb*$^{-/-}$ mice responded to *Nrl* (Fig. 3*C*).

Furthermore, the morphology of photoreceptor nuclei was converted from cone-like in *Rorb*$^{-/-}$ mice to rod-like in *Rorb*$^{-/-}$;*CrxpNrl* mice (discussed below). However, total photoreceptor numbers remained equally reduced in both *Rorb*$^{-/-}$ and *Rorb*$^{-/-}$;*CrxpNrl* mice when counted at P14 (Fig. 3 *D* and *E*). It is possible that *CrxpNrl* expression occurs too late to correct defects in photoreceptor generation or survival in *Rorb*$^{-/-}$ mice or that these actions of *Rorb* are not mediated by *Nrl*.

Transmission electron micrographs showed in detail the recovery of rod-like cells in *Rorb*$^{-/-}$;*CrxpNrl* mice (Fig. 4*A*). In +/+ mice, cones displayed large nuclei with loose chromatin whereas rods displayed smaller and denser nuclei. In *Rorb*$^{-/-}$ mice, all photoreceptor nuclei were cone-like whereas in *Rorb*$^{-/-}$;*CrxpNrl* mice, almost all nuclei had a rod morphology. Photoreceptors in *Rorb*$^{-/-}$ mice lacked inner and outer segments (24). *Rorb*$^{-/-}$;*CrxpNrl* mice possessed many miniature segments (Fig. 4*B*) indicating that the role of *Rorb* in segment formation is mediated in part by *Nrl*.

Photoreceptor Gene Expression Profiles. Microarray analyses showed that most retinal gene expression changes in *Nrl*$^{-/-}$ mice were also represented in *Rorb*$^{-/-}$ mice at P28, confirming the overlap in *Rorb* and *Nrl* functions (Fig. 5*A*). Approximately 80% (100/124) of the genes with $\geq$2-fold increased expression in *Nrl*$^{-/-}$ mice were similarly represented in *Rorb*$^{-/-}$ mice. Almost 80% (104/132) of genes with $\geq$2-fold decreased expression in *Nrl*$^{-/-}$ mice were similarly represented in *Rorb*$^{-/-}$ mice. *Rorb*$^{-/-}$ mice showed greater total numbers of genes with expression changes than did *Nrl*$^{-/-}$ mice in accord with the additional

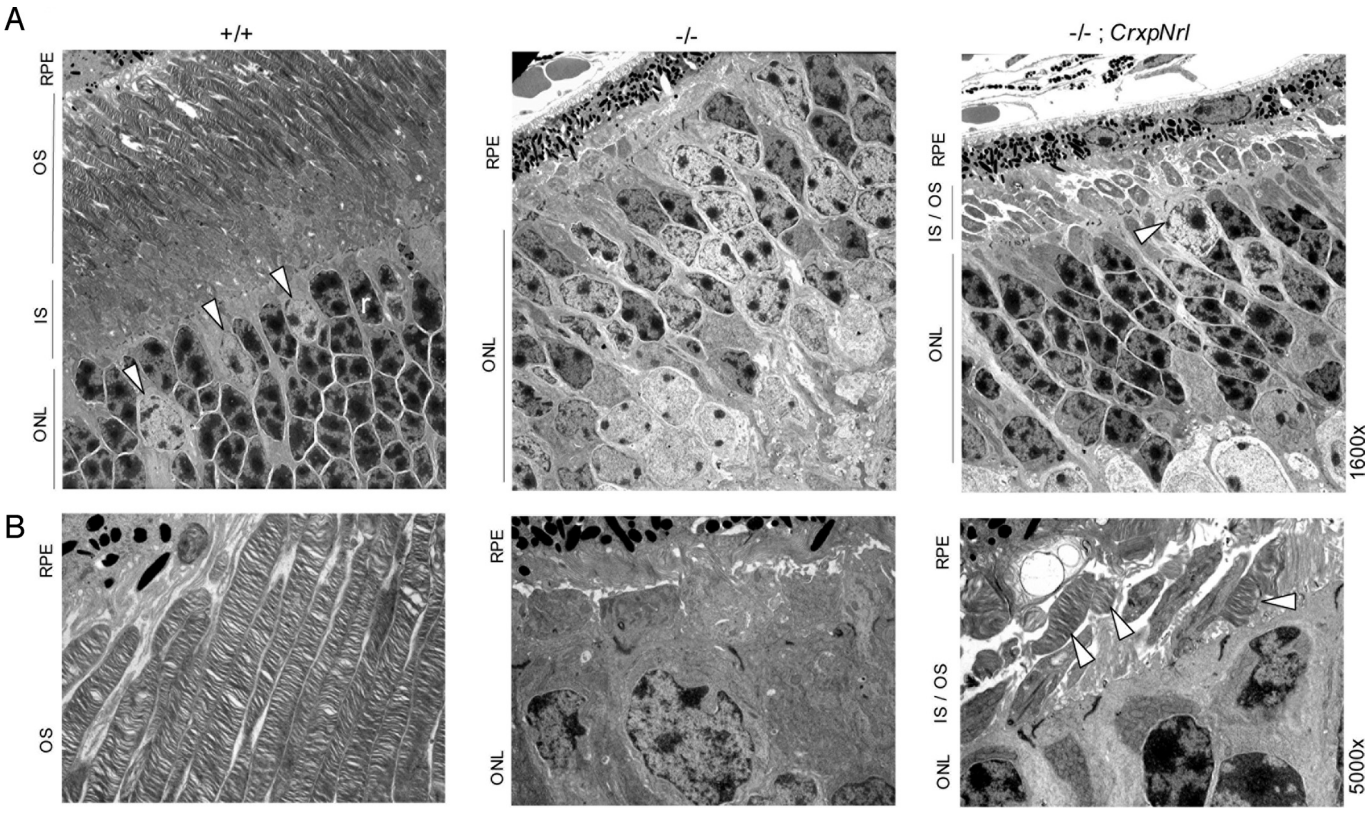

Fig. 4. Rod-like morphology of photoreceptors in *Rorb*$^{-/-}$;*CrxpNrl* mice. (*A*) Transmission electron micrograph of a +/+ mouse retina at P14 showing many compact rod nuclei with dense chromatin (r) and sparser, larger cone nuclei with loose chromatin (arrowheads). In *Rorb*$^{-/-}$ mice, nuclei are cone-like; rod nuclei are absent. In *Rorb*$^{-/-}$;*CrxpNrl* mice nuclei have a rod morphology. A rare remaining cone-like cell is marked (arrowhead, right panel). (*B*), Higher power magnification showing long OS containing stacked disc membranes of +/+ mice (*Left*). *Rorb*$^{-/-}$ mice lack inner and outer segments (IS/OS) (*Middle*) whereas *Rorb*$^{-/-}$;*CrxpNrl* mice have recovered small IS/OS segments (arrowheads)(*Right*).

defects in nonphotoreceptor cell types in *Rorb*$^{-/-}$ mice. It is also possible that these genes include some photoreceptor genes that are under control of *Rorb* but not *Nrl*. Microarray analyses also showed that the *CrxpNrl* transgene in *Rorb*$^{-/-}$ mice partly reversed the expression change of 93% of the genes that were increased in common with *Nrl*$^{-/-}$ mice (Tables S1 and S2). The transgene also reversed expression of 45% of the genes that were decreased in common between the two mutant strains. Of the genes that were altered only in *Rorb*$^{-/-}$ mice, the transgene reversed the expression pattern of relatively few (<17%), consistent with *CrxpNrl* influencing primarily photoreceptors but not other retinal cell types.

Expression changes for selected photoreceptor genes were corroborated by quantitative PCR analysis (Fig. 5*B*). *Rorb*$^{-/-}$ mice displayed decreased expression of rod-related genes but enhanced expression of cone-related genes. The expression changes for most genes examined in *Rorb*$^{-/-}$ mice showed partial reversal in *Rorb*$^{-/-}$;*CrxpNrl* mice in accord with the microarray data.

Discussion

This study indicates that *Rorb* is critical for rod development and that it acts upstream of *Nrl* and *Nr2e3* to control the decision of a photoreceptor precursor to form a rod or cone (Fig. 5*C*). Mutations in *Rorb*, *Nrl*, or *Nr2e3* in mice result in enhanced expression of cone genes at the expense of rod genes reflecting an innate potential of rod precursors to differentiate as cones. Mutation in *Rorb* gives the most severe phenotype of these three genes, with rods being replaced by primitive, nonfunctional cones that lack outer segments. The presence of a small number of rhodopsin^{+} cells in *Rorb*$^{-/-}$ mice that has not been reported in *Nrl*$^{-/-}$ mice may be explained by possible residual *Nrl* expression.

We propose that photoreceptor precursors differentiate by default as cones unless *Rorb* provides an initial impetus to commit to rod differentiation. We suggest that *Rorb* with other unidentified factors promotes the induction of *Nrl*. *Nrl* in turn induces *Nr2e3* and the combined function of these genes fixes the precursor in the rod developmental pathway by inducing rod genes and suppressing cone genes (14). It is possible that *Rorb* also mediates downstream events in rod differentiation, perhaps in cooperation with *Nrl*, *Nr2e3*, or *Crx*. Another newly identified gene in this rod pathway is *Pias3* encoding an E3 SUMO ligase that stimulates the cone suppressing activity of Nr2e3 (29). The *Nrl* gene possesses candidate response elements for nuclear receptors (30), but it is currently unclear if *Nrl* is directly or indirectly induced by *Rorb*.

Rorb is widely expressed in the immature retina, and it evidently serves dual roles in promoting both rod and cone differentiation. In contrast, *Nrl* expression is restricted to rod precursors and, moreover, when ectopically expressed in cones blocks cone differentiation (14). A function for *Rorb* in cones was proposed in previous studies showing that *Rorb* and *Crx* synergistically induce the S opsin gene (*Opn1sw*) promoter during normal cone development (24). It is likely that these independent activities of *Rorb* in rod and cone differentiation are determined in cooperation with other currently unidentified rod- and cone-specific factors that direct regulation of distinct target gene networks in each cell type.

The overexpression of *Opn1sw* in the excess cones in *Rorb*$^{-/-}$ mice is seemingly paradoxical because we previously reported

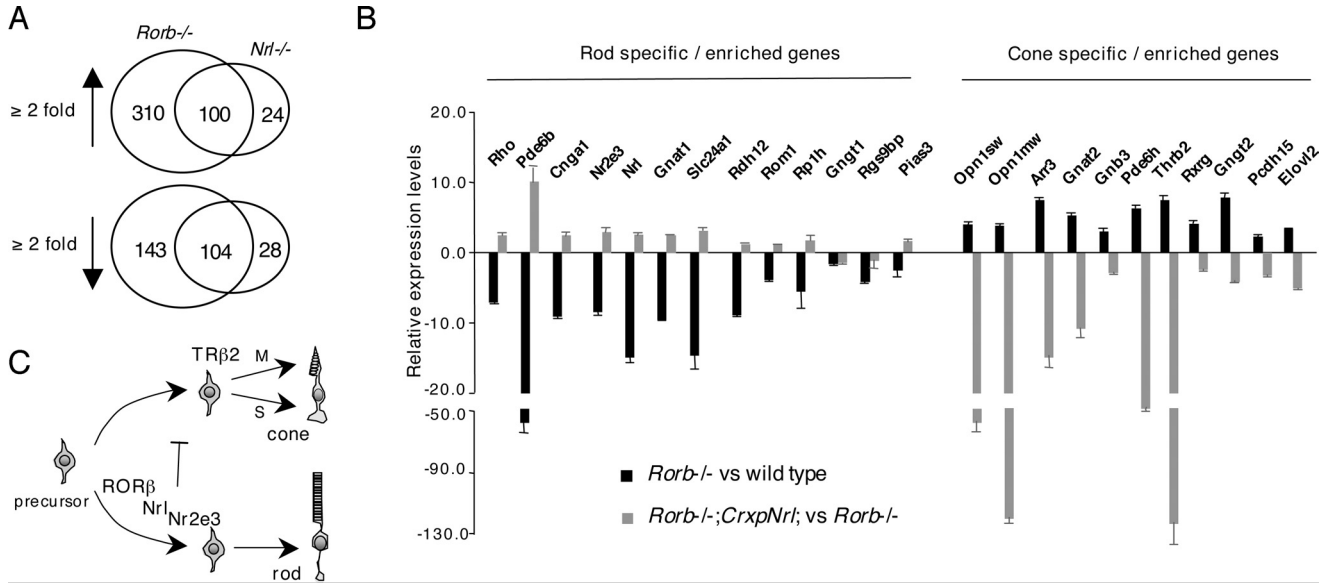

Fig. 5. Retinal gene expression profiles. (*A*) Microarray analysis showing overlap in gene sets with ≥2-fold expression changes in *Rorb*$^{-/-}$ and *Nrl*$^{-/-}$ mice relative to +/+ mice at P28. For overexpressed genes, 100 are common to *Rorb*$^{-/-}$ and *Nrl*$^{-/-}$ mice (out of totals 410 and 124 genes, respectively); 104 under-expressed genes are common to *Rorb*$^{-/-}$ and *Nrl*$^{-/-}$ mice (out of totals 247 and 132, respectively). Unidentified genes were excluded and multiple probes were consolidated for a given gene. (*B*) Quantitative PCR data showing suppressed rod gene and enhanced cone gene expression in *Rorb*$^{-/-}$ mice at P28. *CrxpNrl* partly restores expression of most rod genes and suppresses cone genes. A few under-expressed genes (*Gngt1*, *Rgs9bp*) in *Rorb*$^{-/-}$ mice were not substantially reinduced by *CrxpNrl*. (*C*) Diagram of RORβ, Nrl and Nr2e3 as a hierarchy of factors required for rod differentiation. Loss of these factors allows precursors that would normally form rods to differentiate instead with cone properties. TRβ2 is required for M opsin induction in cones.

that *Rorb* contributes to *Opn1sw* induction in normal cone development (24). However, recent studies show that the same response element in the *Opn1sw* promoter can be activated by RORβ or RORα and that *Rora* mutant mice have reduced *Opn1sw* expression (31). *Rora* may therefore provide a compensatory means of inducing *Opn1sw* in the excess cones in *Rorb*$^{-/-}$ mice. Alternatively, *Crx* alone may suffice to induce *Opn1sw* in the excess cones. The evidence that the excess cones in *Rorb*$^{-/-}$ mice differ from natural cones in allowing *Rorb*-independent induction of *Opn1sw* raises the possibility that the excess and natural cone populations may differ in other subtle ways.

Apart from its role in photoreceptor precursors, *Rorb* may also act in multipotent progenitors as suggested previously by ectopic *Rorb* expression in embryonic rat retina that increased cell clone sizes (23). The precise role of *Rorb* in progenitors is unclear, but it is not essential to generate photoreceptor precursors. However, *Rorb* does influence the rate of generation or survival of these cells because *Rorb*$^{-/-}$ mice ultimately possess only 40% of normal photoreceptor numbers. Like *Rorb*$^{-/-}$ mice, *Otx2*-deficient mice also lack rods but overproduce amacrine-like cells instead of cones (11), revealing a complex interdependence as progenitors are directed toward different retinal cell fates. It is unknown if *Otx2* induces *Rorb* or otherwise promotes rod differentiation. It is possible that the *Rorb-Nrl-Nr2e3* gene hierarchy acts in parallel with the *Otx2-Crx* hierarchy in photoreceptor differentiation, although cross-talk is also likely.

Although the photoreceptor phenotype shows substantial overlap in *Rorb*$^{-/-}$ and *Nrl*$^{-/-}$ mice, subtle distinctions also suggest some different functions for *Rorb*. An example is that overexpressed S opsin retains a normal distribution gradient over the inferior-superior axis of the retina in *Rorb*$^{-/-}$ mice whereas in *Nrl*$^{-/-}$ mice, S opsin is more evenly expressed in all retinal regions (6). A final observation is that *Nrl*$^{-/-}$ and *Nr2e3* mutant mice (7, 6) but not *Rorb*$^{-/-}$ mice form prominent hyperplastic folds in the photoreceptor layer, suggesting that changes in *Rorb*$^{-/-}$ mice preclude the events that cause folding. A possible explanation is that the lower photoreceptor density in *Rorb*$^{-/-}$ mice alleviates folding (32).

Materials and Methods

Details of cDNAs, primers and antibodies are given in Tables S3, S4, and S5.

In Situ Hybridization and Northern Blot Analysis. Eyes were fixed in 4% paraformaldehyde and cryosections were hybridized with digoxigenin-labeled riboprobes as described (33). For Northern blots, 15 μg samples of eye total RNA (7 mice/group) were analyzed using ^{32}P-labeled probes (5).

Western Blot Analysis, Antibodies and Immunohistochemistry. Ten microgram protein samples of nuclear extracts (for Nrl, Nr2e3) or whole cell extracts (other proteins) or 25 μg of whole cell extract (S opsin) were analyzed by Western blot. Bands were quantified by densitometry and normalized to actin (33). Samples were heated at 95 °C before gel-loading only for analysis of Nrl and Nr2e3. Rabbit antiserum was raised against Crx residues 261–274 YSPVDSLEFKDPTG (17) (Covance). For immunofluorescence, 10 μm cryosections were incubated with rabbit anti-TRβ2 or anti-Crx (1:2,500) and Alexa Fluor 568 goat anti-rabbit (1:500), monoclonal anti-βGal (1:200), and Alexa Fluor 488 goat anti-mouse (1:500) antibodies.

Mouse Strains. *Rorb*$^{-/-}$ mice carrying a *lacZ* knockin (22) on a C3H/HeN background were backcrossed for 2 generations onto a C57BL/6J background. The *rd1* mutation was bred out of the C3H background using PCR genotyping (34) with a modified primer (RD6 5′-TACCCACCCTTCCTAATTTTTCTCACGC-3′). *Rorb*$^{+/-}$ mice were crossed to generate +/+, +/− and −/− littermates for analysis. *CrxpNrl* transgenic mice (14) were rederived onto a C57BL/6 × 129/Sv background then crossed with *Rorb*$^{-/-}$ mice. Animal studies followed approved institutional protocols.

Histology and Transmission Electron Microscopy. Eyes were fixed in 3% glutaraldehyde/2% paraformaldehyde. Three micrometer methacrylate sections were stained with hematoxylin and eosin (24). Cells were counted on 160 μm lengths of ONL in the central retina on 3 sections each from 5, 5, and 4 eyes from 3 +/+, 3 *Rorb*$^{-/-}$ and 2 *Rorb*$^{-/-}$;*CrxpNrl* mice, respectively at P14. Electron microscopy was performed on 2 mice/group (JFE Enterprises) (24).

Microarray and Quantitative PCR Analysis. Microarray data are available in the National Center for Biotechnology Information Gene Expression Omnibus

under accession no. GSE16585 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE16585). Microarray analysis was performed on Affymetrix gene chips on 4 independent samples each representing an individual mouse. The experiment was repeated at P28 and P14. Data were compared with data of P28 *Nrl*$^{-/-}$ mice, using a false discovery rate with confidence interval <0.5 as described (35). Real-time PCR was performed twice on triplicate samples of cDNA made from 2.5 μg RNA samples ($\geq$3 mice/group) using SybrGreen I (Molecular Probes) and iCycler IQ PCR detection (Bio-Rad). Average threshold cycle (*C*t) differences were normalized to β-actin control. To assess reversal of gene expression in *Rorb*$^{-/-}$;*CrxpNrl* mice, for overexpressed genes in *Rorb*$^{-/-}$ mice, genes were scored when the ratio of *Rorb*$^{-/-}$ to *Rorb*$^{-/-}$;*CrxpNrl* was $>$1.5-fold. For underexpressed genes, genes were scored when the ratio of *Rorb*$^{-/-}$;*CrxpNrl* to *Rorb*$^{-/-}$ was $>$1.5 fold.

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